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Correction

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Correction for “Two *Cklδ* transcripts regulated by m6A methylation code for two antagonistic kinases in the control of the circadian clock,” by Jean-Michel Fustin, Rika Kojima, Kakeru Itoh, Hsin-Yi Chang, Ye Shiqi, Bowen Zhuang, Asami Oji, Shingo Gibo, Rajesh Narasimamurthy, David Virshup, Gen Kurosawa, Masao Doi, Ichiro Manabe, Yasushi Ishihama, Masahito Ikawa, and Hitoshi Okamura, which was first published May 21, 2018; 10.1073/pnas.1721371115 (*Proc Natl Acad Sci USA* 115:5980–5985).

The authors note that the author name Ye Shiqi should instead appear as Shiqi Ye. The corrected author line appears below. The online version has been corrected.

Jean-Michel Fustin, Rika Kojima, Kakeru Itoh, Hsin-Yi Chang, Shiqi Ye, Bowen Zhuang, Asami Oji, Shingo Gibo, Rajesh Narasimamurthy, David Virshup, Gen Kurosawa, Masao Doi, Ichiro Manabe, Yasushi Ishihama, Masahito Ikawa, and Hitoshi Okamura

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Two *Ck1δ* transcripts regulated by m6A methylation code for two antagonistic kinases in the control of the circadian clock

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The *N*⁶-methylation of internal adenosines (m6A) in mRNA has been quantified and localized throughout the transcriptome. However, the physiological significance of m6A in most highly methylated mRNAs is unknown. It was demonstrated previously that the circadian clock, based on transcription-translation negative feedback loops, is sensitive to the general inhibition of m6A. Here, we show that the Casein Kinase 1 Delta mRNA (*Ck1δ*), coding for a critical kinase in the control of circadian rhythms, cellular growth, and survival, is negatively regulated by m6A. Inhibition of *Ck1δ* mRNA methylation leads to increased translation of two alternatively spliced *CK1δ* isoforms, *CK1δ1* and *CK1δ2*, uncharacterized until now. The expression ratio between these isoforms is tissue-specific, *CK1δ1* and *CK1δ2* have different kinase activities, and they cooperate in the phosphorylation of the circadian clock protein PER2. While *CK1δ1* accelerates the circadian clock by promoting the decay of PER2 proteins, *CK1δ2* slows it down by stabilizing PER2 via increased phosphorylation at a key residue on PER2 protein. These observations challenge the previously established model of PER2 phosphorylation and, given the multiple functions and targets of *CK1δ*, the existence of two isoforms calls for a re-evaluation of past research when *CK1δ1* and *CK1δ2* were simply *CK1δ*.

casein kinase | circadian | methylation | splicing | m6A

Since 2012, the *N*⁶-methylation of internal adenosines (m6A) has regained tremendous interest due to the use of affinity immunoprecipitation of methylated mRNA coupled with next-generation RNA-Seq (1, 2), confirming earlier reports (3, 4). m6A accounts for 50% of all methylated nucleotides in polyadenylated mRNA (5) and for 80% of all base methylations (3). It is the most prevalent internal mRNA modification in higher eukaryotes and is widely conserved from yeast to mammals (6, 7). m6A has been involved in the regulation of mRNA splicing, processing, and translation (8, 9) and is required for survival and development in multiple species (10–12).

The circadian clock is composed of clock genes interlocked in transcription-translation negative feedback loops (TTFL) regulating their own expression (13). This molecular oscillator, robust in normal conditions yet sensitive to perturbations affecting gene expression and RNA processing, allowed us to demonstrate the importance of m6A for its function (14), but which clock genes are directly regulated by m6A remains unknown.

While the core TTFL has been the focus of early circadian research, protein phosphorylation was quickly shown to be essential for molecular timekeeping (15, 16). Phosphorylation of clock proteins is mediated mainly by the Casein Kinases 1 Delta and Epsilon. The first human inherited circadian syndrome to be described, the Familial Advanced Sleep Phase Syndrome

(FASPS), originates from a mutated *CK1δ* (17) or a mutated *CK1δ*-target serine in PER2 protein (18), causing early sleep onset and offset. The first circadian mutation to be discovered in a mammalian model is a mutation in *CK1ε* (16, 19).

Here, we show that the 3'-UTR of *Ck1δ* is heavily m6A-methylated and negatively controls the translation of two *CK1δ* isoforms, uncharacterized until now. These two *CK1δ* isoforms are expressed in a tissue-specific manner and antagonistically regulate the circadian clock.

Results

The 3'-UTR of *Ck1δ* Is Methylated. To identify clock-related transcripts regulated by m6A, we quantified m6A levels in mouse liver mRNA at two time points, CT4 and CT16 (CT is circadian time, and CT0 and CT12 correspond to the beginning of the rest and the active phase, respectively). Significant m6A peaks were found at CT4 (8,414) and at CT16 (8,821). Most peaks (72%) were detected at both time points (Dataset S1). Several transcripts involved in circadian regulation showed significant peaks at CT4 and CT16 (SI Appendix, Fig. S1A). Since *Clock* (20), *Dbp* (21), *Ck1δ* (17), and *Fbxl3* (22, 23) are potent regulators of the

Significance

The most abundant modification in mRNA is the *N*⁶-methylation of internal adenosines (m6A), but m6A's physiological function is unknown for most mRNAs. Here we show that Casein Kinase 1 Delta mRNA (*Ck1δ*), coding for a critical kinase in the control of circadian rhythms, is regulated by m6A. When m6A is inhibited, the expression of two *CK1δ* isoforms, uncharacterized until now, increases due to enhanced translation. This increase in *CK1δ*s leads to a slower clock because of increased phosphorylation of the clock protein PER2 at a key residue, leading to the stabilization of PER2 protein.

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The authors declare no conflict of interest.

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circadian period, m6A methylation of these transcripts is likely to be of importance for clock function. Of these four transcripts, the most significant peak, according to the *q* value of the peak-calling analysis (SI Appendix, Fig. S14), was the single peak in the 3'-UTR of *Ck1δ* (SI Appendix, Fig. S1B), but no significant peaks were detected in the mRNA of its close homolog, *Ck1ε* (SI Appendix, Fig. S1B). Given these observations, together with the relevance of *Ck1δ* for human FASPS and its role as a hub kinase (24), we selected *Ck1δ* for further investigations.

m6A Regulates the Expression of Two CK1δ Isoforms. To investigate the role of m6A in the control of *Ck1δ* expression, we treated mouse embryonic fibroblasts (MEFs) with Deazaneplanocin A (DZnep), an inhibitor of methylation known to inhibit m6A and elongate the circadian period length in these cells (14, 25), and observed an increase in CK1δ proteins (Fig. 1A). This was opposite to our expectations, since available evidence published so far in mammals has associated higher CK1δ expression with a faster clock (26). While replicating these results, we noticed that, under certain conditions during migration of proteins in the acrylamide gel, two bands, not one, were seen on the membrane, both bands increasing under DZnep treatment (Fig. 1A). By comparing these two bands with the bands obtained from cells transfected with CK1δ expression vectors, we later identified the

two bands as two isoforms of CK1δ (Fig. 3), CK1δ1 (415 aa) and CK1δ2 (409 aa), reported in databases but of functions unknown, sharing 99.75% identity with human CK1δs, and conserved in virtually all vertebrates (SI Appendix, Fig. S1 C and D). The only difference between these isoforms is a small 63-bp exon in mouse, retained in *Ck1δ2* immediately upstream of the 3'-UTR and containing a STOP codon (SI Appendix, Fig. S1B), thus coding for the smaller kinase.

To confirm that the results above were dependent upon mRNA m6A inhibition, we silenced in MEFs the catalytic subunit of the mRNA m6A methyltransferase, *Mettl3* (27, 28), and measured CK1δ protein levels by immunoblotting. Again, two specific bands were detected. Silencing of *Mettl3* increased the intensity of the two bands, demonstrating m6A negatively regulates CK1δ expression (Fig. 1B). At the mRNA level, *Mettl3* silencing mildly increased *Ck1δ1* and had little effect on *Ck1δ2* (Fig. 1C) and significantly delayed the initial steps in *Ck1δ1* and *Ck1δ2* degradation (SI Appendix, Fig. S24). Surprisingly, the half-life of *Ck1δ2* was significantly longer than that of *Ck1δ1*, which we confirmed in a longer time course (SI Appendix, Fig. S2B).

Since the increase in CK1δs under m6A inhibition cannot be readily explained by changes in steady-state transcript levels or mRNA stability, we measured the impact of *Mettl3* silencing on translation of *Ck1δ* transcripts by polysome fractionation (Fig. 1D). Consistent with a recent report showing that m6A attenuates

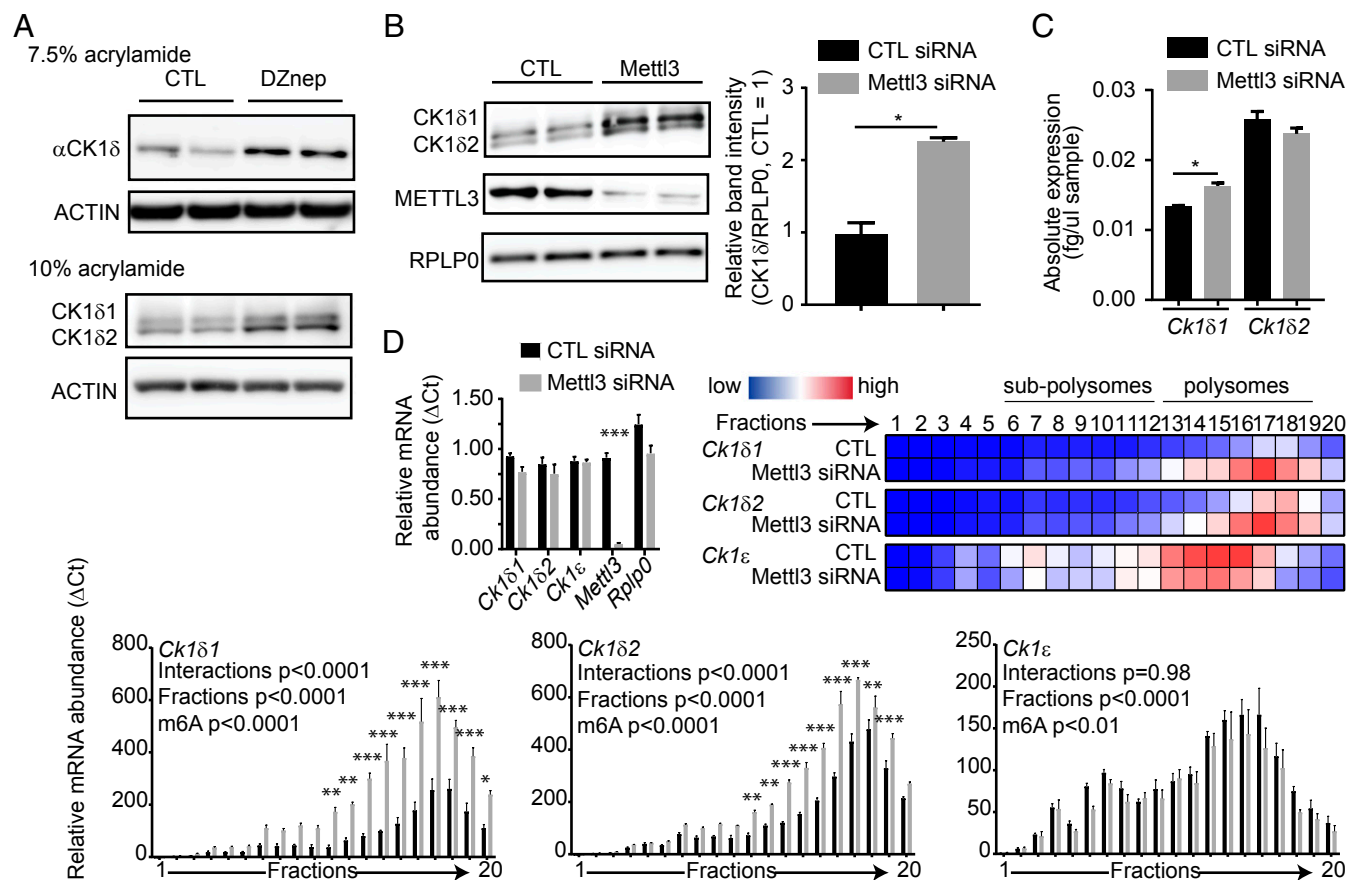


Fig. 1. Two *Ck1δ* alternative transcripts are under m6A control. (A) A 50-μM DZnep treatment for 2 h increased CK1δ protein levels, and two CK1δ isoforms were observed when run on a 10% acrylamide gel. ACTIN was used as a loading control. (B) *Mettl3* silencing increases CK1δ protein levels. Knock-down of *METTL3* was confirmed, and RPLP0 was used as a loading control. (Right) Quantification of the total intensity of CK1δ bands relative to RPLP0, analyzed by Student *t* test, shown as mean ± SD, **P* < 0.05. (C) *Mettl3* silencing causes little changes in *Ck1δ* mRNA steady-state levels. Only *Ck1δ1* showed a mild but significant increase. Data for each transcript were analyzed by Student *t* test and are shown as mean ± SEM, *n* = 3, **P* < 0.05. (D) *Mettl3* silencing increases the translated fraction of *Ck1δ* transcripts. (Top Right) Heatmap visualization of mean relative mRNA abundance for each transcript in each fraction. (Top Left) Quantification of transcripts in input cytoplasmic lysates, showing significant knock-down of *Mettl3* but no significant differences in *Ck1δ1*, *Ck1δ2*, *Ck1ε*, and *Rplp0* mRNA levels; ΔCT data, analyzed by multiple *t* tests, are shown as mean ± SEM, *n* = 3, ****P* < 0.001. (Bottom) Bar charts show mean ΔCT ± SEM of indicated transcripts in each fraction, with significance levels in two-way ANOVA followed by Bonferroni post hoc test, *n* = 3, **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

translation efficiency (29), Mett13 silencing significantly increased the amount of *Ck1δ* transcripts only in fractions containing the heavier polysomes, while *Ck1ε* was not significantly affected.

The 3'-UTR of *Ck1δ* transcripts is over 2,000 nucleotides long, but its function in the regulation of CK1δ expression is unknown. We designed reporter mRNAs in which the luciferase coding sequence was flanked by the 5'- and 3'-UTRs of *Ck1δ*. To closely mimic *Ck1δ1* and *Ck1δ2*, the end of the coding sequence of each *Ck1δ* was also included downstream of the luciferase STOP codon. We also designed *Ck1δluc* transcripts lacking most of the 3'-UTR. These reporter mRNAs were transcribed in vitro and then added to reticulocyte lysates to assess the efficiency of their translation by luminometry (SI Appendix, Fig. S2C). The deletion of the 3'-UTR caused a significant increase in *Ck1δluc* (approximately sixfold) and *Ck1δ2luc* (approximately threefold) luminescence, demonstrating the role of the 3'-UTR as a negative regulator of CK1δ translation.

Ablation of *Ck1δ* m6A Methylation by CRISPR-Cas9 in Vivo. Next, to investigate the physiological role of the methylated locus in the 3'-UTR of *Ck1δ*, we generated a mutant mouse strain with a 43-bp deletion in the genomic region corresponding to the methylated peak (Fig. 2A). This deletion does not affect the coding sequence but removes the N⁶-methylated adenosine at GGm6ACA, located previously by screening of m6A sites in brain and liver transcriptomes (30). The consequences of this mutation on *Ck1δ* m6A levels, mRNA, and protein expression were investigated in the liver, since it is known to have high mRNA m6A levels (2). As expected, *Ck1δ* transcripts had lower m6A levels in -43/-43 animals compared with wild type (Fig. 2B), but the level of *Ck1δ* mRNAs was not significantly affected (Fig. 2B). In contrast, CK1δ protein levels increased significantly: homozygous -43 mutant mice had higher CK1δ levels in the liver and the brain (Fig. 2C), despite an inverted CK1δ1/CK1δ2 ratio (Fig. 3), and had a longer period (23.97 h ± 0.02) of locomotor activity behavior rhythms compared with wild-type mice (23.64 h ± 0.08, Fig. 2D). Although noncoding, this mutation, weakening m6A-dependent posttranscriptional processes limiting CK1δ expression, was sufficient to cause an observable increase in CK1δ proteins, accompanied by a change in circadian locomotor activity behavior. How an increase in the expression of endogenous CK1δs is able to elongate period length is investigated in the next part of this work.

CK1δ1 and CK1δ2 Are Two Different Kinases. Data presented above go against expectations based on period elongation observed in liver explants from *Ck1δ* knock-out mice (31) and on CK1δ overexpression causing a shorter period (26). The overexpression studies, however, did not specify which isoform was used. The existence of two isoforms has been suggested before in a circadian context (32), but has been ignored until now. Surprisingly, since CK1δ1 is considered canonical and has been the de facto focus of past works, CK1δ2 is the major isoform in the liver, where both transcripts show relatively constant levels across 24 h (Fig. 3A). In the suprachiasmatic nucleus of the hypothalamus (SCN), this relationship is reversed (Fig. 3B; see also Fig. 2C), and the molar ratio of *Ck1δ2/Ck1δ1* is tissue-specific (Fig. 3C).

Both CK1δs can interact with PER2 (Fig. 3D), but no evidence of CK1δ1/CK1δ2/PER2 complexes was observed. To test the role of CK1δs in the circadian clock, immortalized MEFs prepared from PER2::LUCIFERASE knock-in reporter mice (33) were stably transfected with CK1δ1 or CK1δ2 expression vectors. Overexpression of CK1δ1 led to a shorter period (Fig. 3E) compared with colonies stably transfected with the empty vector. In contrast, CK1δ2 led to an ~1-h period elongation. While both isoforms could phosphorylate PER2 (Fig. 3F), the intensity of the phosphorylated bands was higher with CK1δ2 and CK1δ1 + CK1δ2, suggesting higher kinase activity for CK1δ2 and/or faster PER2 degradation with CK1δ1. A picture emerges in which CK1δ isoforms may not simply be two redundant kinases but may phosphorylate PER2 differently and in cooperation.

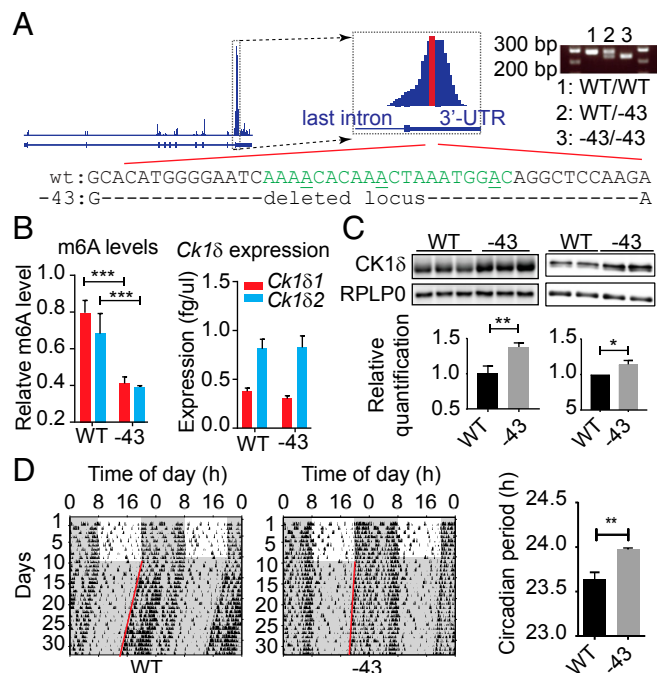


Fig. 2. Deletion of m6A locus from *Csnk1d* 3'-UTR is sufficient to elongate the circadian period in vivo. (A) Location of the mutation and genotyping of mice bearing a -43 deletion in the *Csnk1d* gene. The region of the zoomed m6A peak highlighted in red corresponds to the peak summit identified by MACS2 peak calling analysis. (B) Quantification of *Ck1δ* mRNA m6A levels (Left) and of *Ck1δ1* and *Ck1δ2* mRNA expression in the liver by qPCR (Right). Analyzed by two-way ANOVA (wild type vs. -43, $P < 0.0001$, $n = 3$) followed by Bonferroni post hoc, $***P < 0.001$. (C, Left) Representative immunoblotting for CK1δ and loading control RPLP0 with liver samples. Mean ± SEM quantification of CK1δ band intensity analyzed by Student t test is shown below; $n = 3$, $***P < 0.01$. (C, Right) Representative immunoblotting for CK1δ and loading control RPLP0 with $n = 2$ brain samples. (Bottom) Mean ± SEM quantification of data aggregated from three independent experiments for a total of $n = 6$ animals, analyzed by Student t test; $*P < 0.05$. (D) Circadian locomotor activity recording from one representative animal of each genotype is shown. (Right) Period was analyzed by χ^2 periodogram; mean period ± SEM was analyzed by Student t test of $n = 6$ animals.

Work on CK1δ and -ε have shown that PER2 phosphorylation can either accelerate or decelerate the clock depending on which residue is targeted (15, 34, 35). pS478 in mice leads to faster degradation of PER2 by the ubiquitin ligase β-Trep (36–38), while pS659, named FASPS because its mutation in humans (S662G) causes FASPS, stabilizes PER2 and elongates period (16, 18, 34, 38–40). S659 and three to four nearby downstream residues are phosphorylated in vivo, but work in vitro using CK1ε or CK1δ has shown that CK1 needs a priming phosphorylation at S659 by an unknown “priming” kinase before phosphorylating downstream residues in the cluster, which is necessary for PER2 stabilization. In vitro experiments concluding that CK1δ could not phosphorylate S659, however, used a truncated CK1δ containing only the N-terminal kinase domain and thus gave no insights into the respective functions of CK1δ isoforms (41).

CK1δ1 and CK1δ2 Lead to Different PER2 Phosphorylation Patterns. To gain insights into the respective functions of CK1δs, we refined a previously published mathematical model of the regulation of PER2 by branched phosphorylation fates (38) to allow the rate of each phosphorylation step to be independently tuned (SI Appendix, Fig. S3A). As explained in SI Appendix, Supplemental Results, our model predicted that CK1δ2 could be the priming kinase. To test this possibility, we transfected PER2::LUC MEFs with the vector mixes used in Fig. 3E, immunoprecipitated MYC-PER2, and

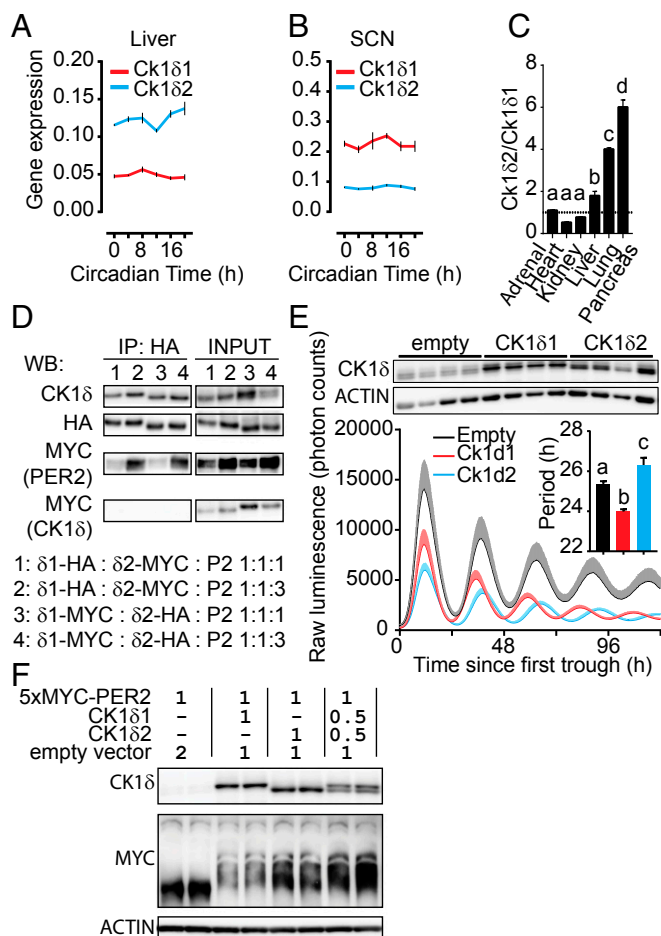


Fig. 3. CK1δ1 and CK1δ2 are antagonistic. *CK1δ1* and *CK1δ2* mRNA levels in the liver (A) and the SCN (B) across a circadian day, with opposite *CK1δ2/CK1δ1* ratios mainly due to different levels of *CK1δ1*. Mean \pm SEM analyzed by two-way ANOVA, $n = 3$, significant for *CK1δ1* vs. *CK1δ2* ($P < 0.0001$) and for time, only in the liver for *CK1δ2* ($P < 0.05$). (C) Tissue-specific *CK1δ2/CK1δ1* ratios measured in six different tissues. Analyzed by one-way ANOVA, $P < 0.0001$, followed by Bonferroni post hoc, a vs. b vs. c vs. d , indicating at least $P < 0.05$ significance. Results shown are mean \pm SD of $n = 2$. (D) Coimmunoprecipitation shows that both CK1δ1-HA and CK1δ2-HA interact with 5xMYC-PER2. Two CK1δ1-tag:CK1δ2-tag:5xMYC-PER2 ratios were used at 1:1:1 and 1:1:3. (E) Stable overexpression of CK1δ1 and CK1δ2 in PER2::LUC MEFs leads to period shortening and lengthening, respectively. (Inset) Period analyzed by one-way ANOVA, $P < 0.001$, with a vs. b vs. c in Bonferroni post hoc at least $P < 0.05$; results shown are mean \pm SEM of $n = 4$ monoclinal cell lines for each group. (Top) CK1δ and ACTIN immunoblots for each cell line. (F) Cotransfection of 5xMYC-PER2 with untagged CK1δ1 and/or CK1δ2 shows higher 5xMYC-PER2 phosphorylation (and/or higher stability) when CK1δ2 is present.

quantified PER2 phosphopeptides after trypsin digestion. Tetra-, tri-, di-, and unphosphorylated FASPS peptides were detected (Fig. 4A, SI Appendix, Fig. S4, and Dataset S2). The amount of unphosphorylated FASPS peptide was highest without CK1δs, and lowest with CK1δs together. The diphosphorylated peptide, always detected without pS659, had similar levels with or without CK1δs. The level of triphosphorylated peptides showed some variations between replicates but was always highest when both CK1δ1 and CK1δ2 were present. Remarkably, the tetraphosphorylated FASPS peptide, with confirmed pS659 (SI Appendix, Fig. S4B), showed the most pronounced isoform-dependent differences in abundance among all PER2 phosphopeptides detected (SI Appendix, Fig. S4A and Dataset S2). It was undetected without CK1δ

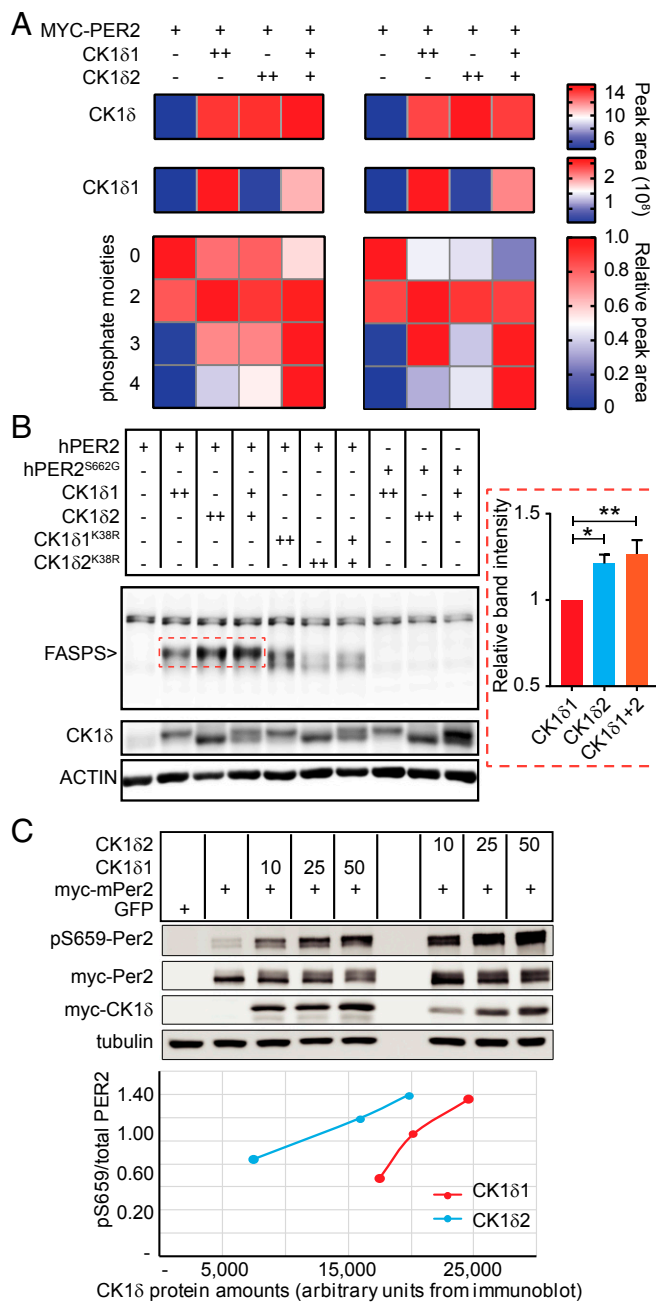


Fig. 4. CK1δ1 and CK1δ2 cooperate in phosphorylating PER2. (A) CK1δ isoform-dependent PER2 FASPS phosphopeptides analysis. Data from two technical replicates are provided, side by side. CK1δ peptides were also detected: the first row shows total CK1δ, and the second row corresponds to the CK1δ1-specific VASSGLQSVVHR peptide. The 4×4 heatmaps show quantification of the FASPS peptide, sorted by number of phosphate moieties detected. The bar on the Right is the color legend for each corresponding heatmap. (B) Immunoblotting of hPER2 FASPS phosphorylation by CK1δ isoforms. CK1δ and actin immunoblotting are shown as reference and loading control, respectively. Graph on the Right shows mean FASPS band intensity \pm SEM of $n = 4$ independent replicate wild-type hPER2 transfections with CK1δ1, CK1δ2, and 1 + 2. $*P < 0.05$; $**P < 0.01$. (C) mPER2 was expressed in HEK293 cells in the presence of increasing amounts of vectors (10, 25, 50 ng) encoding CK1δ splice variants as indicated. Forty-eight hours after transfection, lysates were probed with anti-pS659 mPER2, anti-Myc, and anti-tubulin antibodies. (Bottom) The ratio of pS659/total PER2 as a function of CK1δ amounts.

isoforms increases, affecting the circadian clock. It is likely, given that *Dbp*, *Fbxl3*, and *Clock* transcripts are also methylated, that global inhibition of mRNA m6A methylation would still affect the clock in $-43/-43$ animals, as the general disruption of mRNA processing caused by the lack of m6A would still occur.

While the lack of CK1 δ is embryonic lethal (31), higher expression or increased activity has been linked to cancer progression, migraine, and circadian disruption (24). As we demonstrated here, the expression of CK1 δ isoforms is kept to a low level by its methylated 3'-UTR. The significance of these observations goes beyond the circadian field, given the manifold functions of CK1 δ . Further investigations should reveal what the respective physiological functions of CK1 δ isoforms are, and additional discussion on the role and regulation of CK1Ds can be found in *SI Appendix, Supplemental Discussion*.

Materials and Methods

Reagents were from Nacalai Tesque unless stated otherwise.

Animals. All experiments were approved by the animal experimentation committee of Kyoto University. Twelve-week-old C57BL/6 males were maintained at $23^{\circ}\text{C} \pm 1^{\circ}\text{C}$ with $50\% \pm 10\%$ relative humidity, on a 12-h light/12 h dark cycle (lights on 8:00, lights off 20:00), food and water ad libitum. For Fig. 3 A and B, lights were permanently switched off from 8 PM on day 0, and animals were sampled from 8 AM (corresponding to circadian time 0) on day 2.

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